Prevalence and causes of abnormal PSA recovery

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Abstract: BACKGROUND: Prostate-specific antigen (PSA) test is of paramount importance as a diagnostic tool for the detection and monitoring of patients with prostate cancer. In the presence of interfering factors such as heterophilic antibodies or anti-PSA antibodies the PSA test can yield significantly falsified results. The prevalence of these factors is unknown. METHODS: We determined the recovery of PSA concentrations diluting patient samples with a standard serum of known PSA concentration. Based on the frequency distribution of recoveries in a pre-study on 268 samples, samples with recoveries <80% or >120% were defined as suspect, re-tested and further characterized to identify the cause of interference. RESULTS: A total of 1158 consecutive serum samples were analyzed. Four samples (0.3%) showed reproducibly disturbed recoveries of 10%, 68%, 166% and 4441%. In three samples heterophilic antibodies were identified as the probable cause, in the fourth anti-PSA-autoantibodies. The very low recovery caused by the latter interference was confirmed in serum, as well as heparin- and EDTA plasma of blood samples obtained 6 months later. Analysis by eight different immunoassays showed recoveries ranging between <10% and 80%. In a follow-up study of 212 random plasma samples we found seven samples with autoantibodies against PSA which however did not show any disturbed PSA recovery. CONCLUSIONS: About 0.3% of PSA determinations by the electrochemiluminescence assay (ECLIA) of Roche diagnostics are disturbed by heterophilic or anti-PSA autoantibodies. Although they are rare, these interferences can cause relevant misinterpretations of a PSA test result.

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Results: A total of 1158 consecutive serum samples were analyzed. Four samples (0.3%) showed reproducibly disturbed recoveries of 10%, 68%, 166% and 4441%. In three samples heterophilic antibodies were identified as the probable cause, in the fourth anti-PSA-autoantibodies. The very low recovery caused by the latter interference was confirmed in serum, as well as heparin- and EDTA plasma of blood samples obtained 6 months later. Analysis by eight different immunoassays showed recoveries ranging between <10% and 80%. In a follow-up study of 212 random plasma samples we found seven samples with autoantibodies against PSA which however did not show any disturbed PSA recovery.

Conclusions: About 0.3% of PSA determinations by the electrochemiluminescence assay (ECLIA) of Roche diagnostics are disturbed by heterophilic or anti-PSA autoantibodies. Although they are rare, these interferences can cause relevant misinterpretations of a PSA test result.

Keywords: autoantibodies; heterophilic antibodies; immunoassay; prostate-specific antigen (PSA); prostate; recovery.

Introduction

Prostate-specific antigen (PSA) is a very important biomarker for the detection, staging and monitoring of patients with prostate cancer [1, 2]. Elevated PSA levels may lead to diagnostic interventions (i.e. prostate biopsy [3]) or in some cases even trigger additional therapies (i.e. androgen deprivation or salvage radiation) for patients with known prostate cancer [4, 5]. Hence, a PSA immunoassay must provide accurate and reproducible results as they directly influence important clinical decisions.

Immunoasssays using monoclonal antibodies are generally robust but may be disturbed by interferences such as heterophilic antibodies (hAs) or auto antibodies [6, 7]. Several incidents of falsely elevated PSA levels as the result of hAs have been reported [8–10] and in some cases this led to severe overtreatment of patients [11, 12]. Due to the fact that these interferences are widely unknown in the urologic community, it can be assumed that there is a relevant number of unknown cases. Also auto antibodies against PSA have been described to occur in men with prostate cancer, benign prostate hypertrophy or immunoinfertility at prevalences ranging between 3% and 30% [13, 14]. At least in theory PSA-autoantibodies can result in false-low levels of PSA and subsequently underdiagnosis and undertreatment of incident or relapsing prostate cancer. As yet the prevalence of these interferences has not been systematically investigated [15, 16].

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This is in contrast to the monitoring of thyroid cancer by thyroglobulin (TG) [17], after thyroidectomy and radiation [18]. International guidelines mandate that every TG test must be accompanied by the measurement of anti-TG antibodies and/or the recovery of TG added to the sample [19].

In this study we investigated the prevalence and underlying reasons for disturbed PSA measurements in a large consecutive series of serum samples.

Materials and methods

Study design

From February to May 2014 all sera which were sent to the Institute for Clinical Chemistry for a PSA measurement were routinely analyzed for recovery. As the first step, a pilot study was performed on 268 samples to determine the frequency distribution of recoveries. The 99.7% confidence interval was defined as the normal range and ranged from 80% to 120%. As the second step 1158 consecutive samples were screened to identify samples with recoveries of less than 80% or more than 120%. Pathological samples were re-analyzed for recovery and by scantibodies to identify samples with reproducibly abnormal recovery and hA, respectively. Finally we approached patients with reproducibly abnormal recovery to obtain novel blood samples. Figure 1 shows the flowchart of the conducted study.

Both the screening study and the patient characterization were approved by the Local Ethics Committee.

Quantification of PSA concentrations and PSA recoveries

PSA was determined by a 3rd generation electrochemiluminescence assay (ECLIA) from Roche diagnostics using the e601 unit of

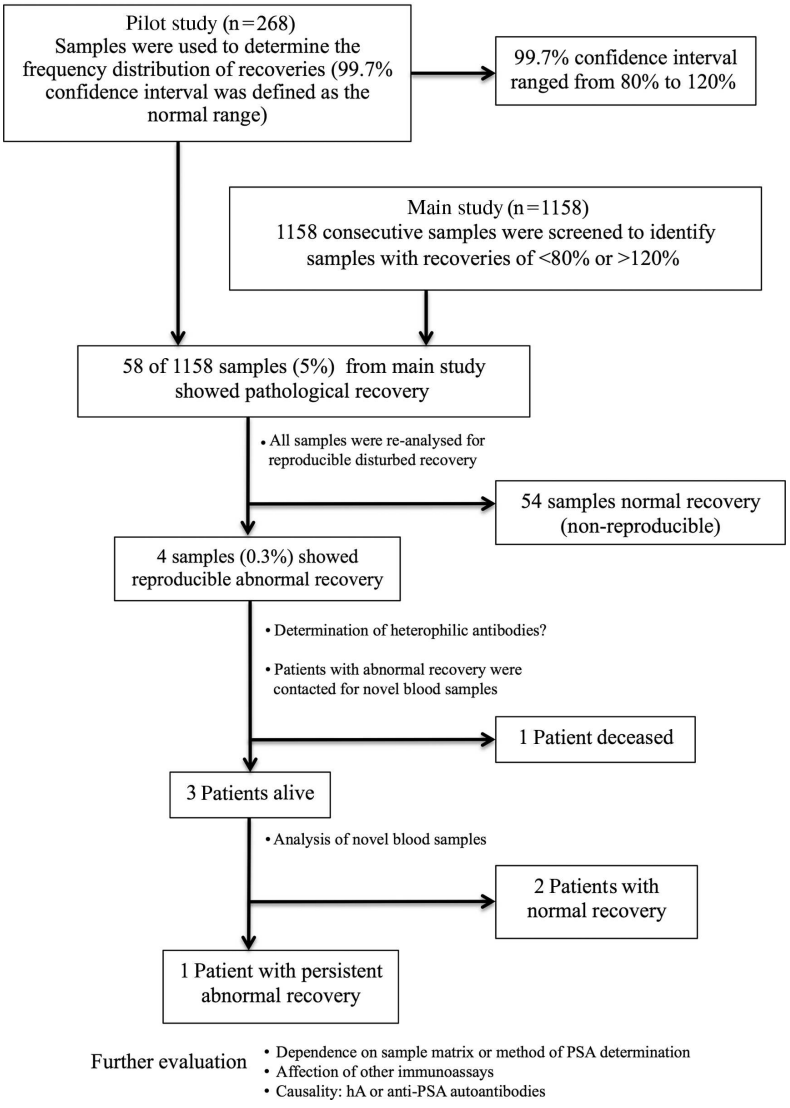

Figure 1: Study flow chart of the conducted study.

a COBAS8000 system (Rotkreuz, Switzerland). To determine recoveries, we mixed 190 µL of original patient samples with 10 µL of a sample from a patient with a very high PSA level (Pool) level of 128 ± 5 µg/L. Recovery was defined as the ratio of observed PSA concentrations to expected PSA concentrations in the mixed samples (observed PSA/expected PSA in %). The expected PSA concentration was calculated the following: µg/L PSA in pool serum*10 µL + µg/L PSA in patient serum*190 µL)/200 µL.

As the result of the pilot study, recoveries of <80% or >120% were defined as abnormal.

PSA recovery of one specific sample was also analysed by using eight different PSA immunoassays in collaborating laboratories (see acknowledgements).

Heterophilic antibodies

Sera with disturbed recovery were incubated with a blocking reagent for hAs (Scantibodies Heterophilic Blocking Reagent; Scantibodies Laboratory Inc., Santee, CA, USA) before PSA was determined. In 39 randomly selected sera (14 frozen, 25 fresh) we assessed the concentrations of PSA before and after incubation with the blocking reagent and thereby found recoveries of 96% + 9.5%. We therefore defined a range of 67%–124% ($\mu \pm 3\sigma$) as non-pathologic absorbance.

Anti-PSA autoantibodies

Anti-PSA autoantibodies were detected by indirect ELISA against purified PSA-antichymotrypsin (ACT) complex (provided by Roche diagnostics, Penzberg, Germany), ACT, and free PSA (Sigma).

Briefly, PSA-ACT, free PSA or ACT in 100 mM carbonate-bicarbonate buffer pH 9.6 were bound to polystyrene microtiter plates overnight at 4 °C, followed by extensive washing. The wells were then incubated with blocking buffer (2% BSA in PBS 0.1% Tween20) for 2 h at room temperature. After a second washing step, plasma samples diluted in blocking buffer were then added to each well. The plate was incubated under these conditions at 4 °C overnight on a plate shaker. A third washing step was then performed before an HRP-conjugated antibody against human IgG (Jackson ImmunResearch) diluted in blocking buffer was added for 2 h at room temperature. After a further washing step, HRP activity was detected by using an ABTS substrate solution (Roche). Absorbance was read at 405 nm with an automated plate reader (Tecan, Biotek). The absorbance at 490 nm was used as a reference. All washing steps were carried out using an EL406 washer-dispenser (Biotek). The washing buffer employed was PBS 0.1% Tween20. The plasma samples and the anti-IgG antibody were diluted as reported in the figure legends.

Detection was performed using an HRP-conjugated polyclonal rabbit anti-mouse secondary antibody (Dako). The anti-PSA-antibody search test was developed only for this research study and is not part of our clinical routine diagnostics.

Anti-PSA-ACT autoantibodies in plasma were also detected by western blotting. Briefly, 1 µg of pure PSA-ACT was separated on a 10% SDS-PAGE gel in reducing conditions followed by transfer to a nitrocellulose membrane. Detection was performed using the Pierce ECL Plus western blot substrate (Thermo Scientific) on a Fusion FX chemiluminescence imaging station (Viber Luormat).

For further detection of anti-PSA autoantibodies, immunohistochemistry on prostate cancer cells was performed. The human prostate cancer cell-line PC3 [20] were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described [21]. Cells were stained using the serum of the patient as a primary antibody and anti-human IgG as a secondary antibody. Immunohistochemistry was carried out on a Leica Bond immunostainer (Leica Microsystems, Newcastle, UK) according to the manufacturer's instructions.

Results

Discovery studies

The pilot study on 268 samples revealed a mean recovery of 104% with a standard deviation (SD) of 7% and a ±3 SD ranging from 83% to 125%. We therefore defined recoveries of less than 80% or more than 120% as pathological. A prevalence of 5% off all samples showed initial recoveries outside of this range (Figure 1). Re-testing of these suspect samples confirmed pathological recoveries in four samples: 10% (Patient 1), 68% (Patient 2), 165% (Patient 3), and 4441% (Patient 4). (Table 1 and Supplemental Figure 1). Thus, four out of 1158 samples showed reproducibly pathological recoveries (prevalence of 0.3%).

To identify the reason for the interferences we first checked the samples for the presence of hA by using the adsorber test from Scantibodies. Three samples showed strongly pathological recoveries of 826,300% (Patient 4), 3123% (Patient 3), and 2% (Patient 2). The sample of Patient 1 showed a borderline pathological recovery of 63% (Table 1).

Case reports and validation studies

The four patients with reproducibly abnormal recovery were invited to provide novel blood samples to test for persistence of the interference, the impact of the sample matrix and the presence of anti-PSA autoantibodies (Figure 1). In addition, an extended clinical history was recorded to identify exposures that may explain the presence of hA (Table 2). As Patient 4 had died in the meantime, only three of the four patients could be contacted.

The PSA of Patient 1 was requested as part of a routine check up. He had a history of prostatoepididymitis and had received one blood transfusion. Patient 2 suffered from lower urinary tract syndrome (LUTS), but also had pure red cell aplasia. For this reason he was exposed to many blood transfusions as well as with one human

Table 1: Concentrations and recoveries of PSA in sera of four patients with reproducibly disturbed PSA recovery.

Patient	Initial serum sample							2nd serum sample				
	Original PSA, ng/mL	Pool	Expected PSA, ng/mL	Measured mix PSA, ng/mL	Recovery (%)	PSA value after hA absorbance, ng/mL	Recovery after hA absorbance (%)	Measured PSA, ng/mL	Pool	Expected PSA, ng/mL	Measured Mix PSA, ng/mL	Recovery (%)
1	0.19	124.9	6.43	0.66	**10.3**	0.12	63.2	0.16	123.9	6.35	0.55	**8.7**
2	5.00	135.3	11.52	7.87	**68.3**	0.08	1.6	3.11	123.9	9.15	8.76	95.7
3	0.13	135.3	6.89	11.36	**164.9**	4.06	3123.1	0.15	123.9	6.34	6.65	104.9
4	0.03	124.9	6.27	278.60	**4440.9**	247.90	826333.3	n.a.	n.a.	n.a.	n.a.	n.a.

Bold face representing pathological values outside the predefined recovery range (< 80% or > 120%).

Table 2: Clinical findings of patients with reproducibly disturbed PSA recovery.

Patient	Reason for disturbed PSA recovery	Age, years	Environment		Medical history				
			Profession	Contact to animals or animal products (including pets)	PCa	History of tranfusions or immunotherapies	Medication	Szintigraphy	Childhood illnesses
1	PSA-aA	50	Auto mechanic	Fish, dog, cats, birds	No	EC transfusion	Tamsulosin	Yes	Varizella, herpes
2	hA	67	Metal worker	Goats, chicken, cats, dogs	No	EC and IG transfusion	Ciclosporin	Yes	Unclear
3	hA	57	Plumber	Horses, chicken, cows, sheep	No	No	Metformin, statin, tamsulosin, ace inhibitor	No	Rubella
4	hA	76	n.a.	n.a.	Yes	EC transfusion	n.a.	n.a.	n.a.

PSA, prostate-specific antigen; PCa, prostate cancer; hA, heterophilic antibodies; PSA-aA, PSA auto-antibodies; EC, erythrocyte concentrate; IG: immunoglobulin.

Table 3: PSA recoveries as determined in the serum of Patient 1.

	Roche Cobas	Abbott Architect	Siemens Advia Centaur	Beckman Hybritech	Beckman Access 2	Siemens Immulite	i-chroma	Biomerieux mini Vidas	Siemens Dimension Vista
Measured PSA, ng/mL	0.16	0.15	1.72	2.77	3.4	3.18	0.1	0.29	0.08
Recovery, %	9	16	57	66	80	80	6	15	6

immunoglobuline infusion (KIOVIG™). Patient 3 was suffering from LUTS and had no history of any exposure to any transfusion. Patient 4 had suffered from castration resistant prostate cancer at the time point of first sample testing. He died shortly thereafter as a result of his metastatic disease. He had a history of blood tranfusion.

PSA concentrations and recoveries were determined in the newly obtained blood samples from the three living patients (Table 1). Interestingly, recoveries were normal in sera of Patients 2 and 3, but severely decreased to less than 10% in Patient 1 no matter whether PSA was quantified in serum, EDTA-plasma or Li-Heparinate-plasma. As a further indication of the interference the free PSA concentration (2.26 µg/L) was much higher than the apparent total PSA concentration (0.16 µg/L). The recovery of PSA was found variably reduced upon quantification by different immunoassays from different manufacturers: Four assays showed borderline to intermediate recoveries of 57%–80%, whereas four other assays showed severely reduced recoveries of 5%–16% like the Roche Cobas assay (Table 3).

Finally we tested the sample of Patient 1 for the presence of anti-PSA antibodies by indirect immunostaining of PC3 prostate cancer cells, western blotting, and ELISA. Immunohistochemistry of PC3 using the serum of Patient 1 confirmed the presence of anti-PSA antibodies in the patient serum. PC3 prostate cancer cells showed a dose dependent positive PSA-like immunoreactivity when the patient serum was used a primary antibody (Figure 2A–C). Western blotting of purified PSA with the patient serum but not with control serum identified immuno-reactivity against the PSA-ACT-antigen used in the total PSA assay. The purity, integrity and molecular weight of the PSA-ACT complex were confirmed by SDS-PAGE followed by Comassie staining (Figure 2D). By ELISA, we found 1:10 and 1:5 dilutions of the patient plasma as anti-PSA-immunoreactive (Figure 3A). Plasma samples from 212 randomly selected male patients from the sample archive of the institute of clinical chemistry identified seven samples with significant immunoreactivity against the PSA-ACT antigen (Figure 3B). However, all of them showed normal recoveries both of total PSA and free PSA. Therefore we analysed the immunoreactivity of the plasma of Patient 1 as well as the plasmas of the seven patients with immunoreactivity against the ACT/PSA complex for their immunoreactivity against free PSA, ACT, and the ACT-PSA complex purchased from Sigma (Figure 3C). The disturbed sample of Patient 1 showed immunoreactivity against both the pure and ACT-complexed PSA but none against ACT. The immunoreactivity pattern of the other seven samples was more heterogenous: Samples A, C, D, E, and G immunoreacted with all three antigens whereas samples B and F reacted with only free PSA but not with complexed PSA or ACT (Figure 3C).

Discussion

To our knowledge this is the first systematic study on the prevalence and underlying reasons of disturbed PSA recovery in a large consecutive series of clinical serum samples. We determined recoveries by patients' plasmas with a small amount of a pool plasma with very high PSA concentration. The spiking plasma hence accounted for only 5% of the final sample. This very low amount makes artificial interferences by the spiking plasma unlikely. In line with this the frequency distribution of recoveries ranged from 80% to 120% both in the pilot and the validation. Any interference by the spiking plasma should have resulted in a frequency distribution that is skewed to values either lower or higher than 100%.

Among 1158 sera we identified four samples (0.3%) with reproducibly pathological recoveries. However, the interference was found to persist in only one sample over time when samples were retested several month later. This persistent interference was found to be associated with the presence of anti-PSA auto-antibodies whereas the non-persistent interference was associated with the presence of hA.

The prevalence of hA in serum samples was reported to vary from 3% to 40% [16, 22]. However, clinically more important is the prevalence of disturbed PSA levels due to hA in routine PSA assessment. In line with our results, Anderson et al. [15] estimated the prevalence of spuriously elevated PSA values in patients after curative treatment

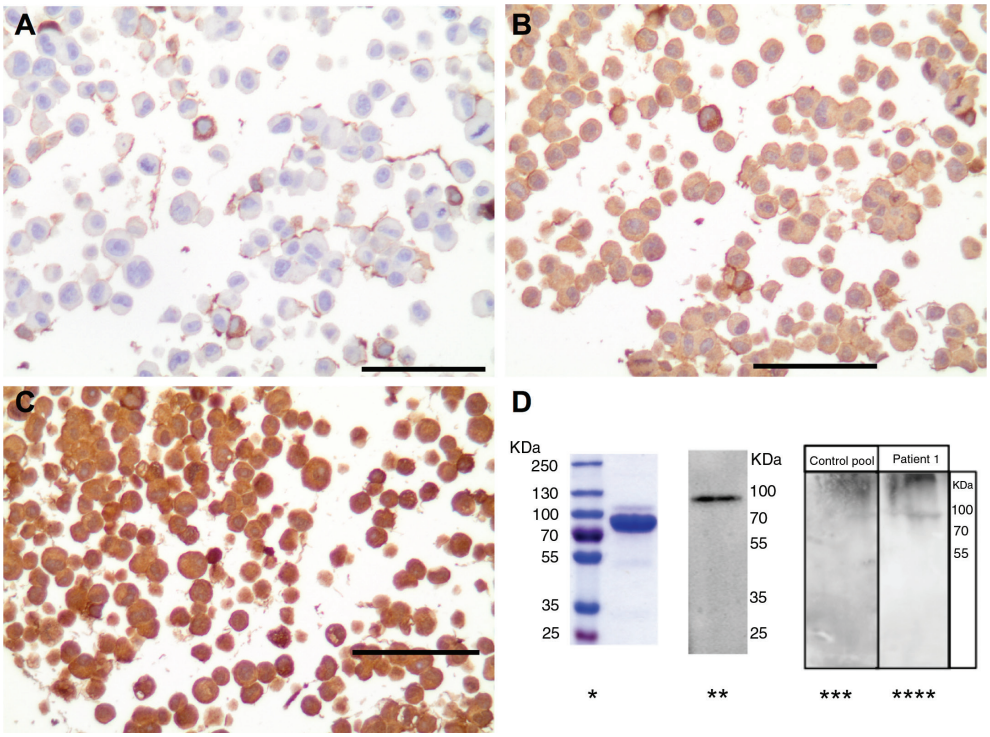

Figure 2: Demonstration of the anti-PSA antibody in the patient serum by Immunohistochemistry of formalin-fixed paraffin-embedded PC3 prostate cancer cells (A–C) and Western Blotting (D).
(A) Negative control (without patient serum, IgG was used as secondary antibody). (B) Serum of the patient was used instead of a primary antibody (concentration 1:500). (C) Serum of the patient was used instead of a primary antibody (concentration 1:100). (D) A purified PSA-ACT complex was electrophoresed by SDS-PAGE and stained with Comassie blue (*) or transferred to nitrocellulose for immunodetection by a mouse monoclonal anti-PSA antibody (**), a serum pool (***) or the serum of Patient 1 (****).

for prostate cancer in their study to be 0.3%. However, the tested population was highly selected (only patients after curative treatment for prostate cancer) and cannot be directly compared with ours. Our study was performed on unselected samples of male patients, who underwent PSA testing for different reasons, i.e. screening or urological work-up for suspected prostate cancer, monitoring of treatment for prostate cancer. Moreover, our reported prevalence is not estimated but determined.

HA are human immunoglobulins that bind against different animal antigens, for example from mice. Interference of hA with immunoassays has been known since the 1980s [7]. Most PSA assays including ours from Roche diagnostics use a solid-phase immunoglobulin G to bind the PSA molecule. A second immunoglobulin, marked with a tracer, binds subsequently to the PSA molecule for measurement. As hAs can bind to either immunoglobulin, the presence of hAs can lead to false-positive or, more rarely, false-negative results by cross-binding the assay antibodies [7, 23].

Usually, the reason for the presence of a hA in a serum remains unkown. Samples of patients formerly exposed to animals or animal serum products, immunoglobulin therapy or immunoscintigraphy are more likely to contain hAs [24]. HAs can also result from infections by rubella, measles, adeno-, entero-, and varicella-zoster viruses [25]. Our patients show several of these risk factors. Of note, the interference with hAs did not persist over time but disappeared in samples newly obtained several months after the initial sample. Also in the patient of our previous case-report [10], we observed disappearance of the interference during a follow-up of several months (Poyet et al. unpublished).

Previously, interference of PSA assays by hA became obvious by falsely elevated PSA values during screening [8, 11, 26], persistence of elevated PSA [10, 12, 27, 28] or PSA recurrence after curative treatment [9, 29]. A relevant amount of these reported cases led to unnecessary salvage treatments or diagnostic investigations for metastasis.

Anti-PSA autoantibodies were found in one patient as the cause of severely decreased PSA recovery. Of note, the autoantibodies only interfered with the determination of total PSA but not free PSA. A finding of free PSA

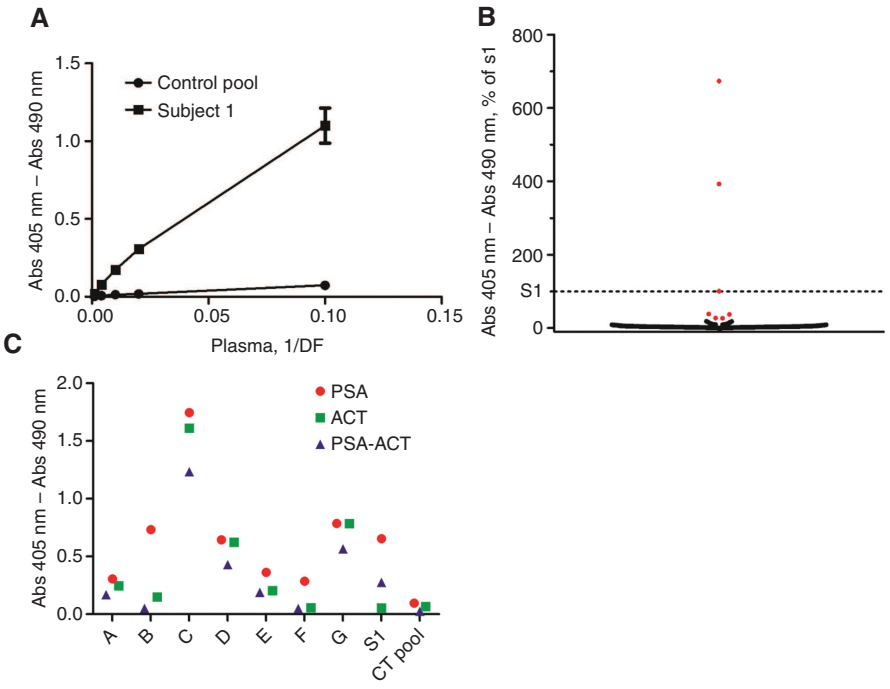

Figure 3: Detection of autoantibodies against PSA, PSA-ACT and ACT.
(A) Indirect ELISA against PSA-ACT. Comparison between Subject 1 and a control plasma pool. Data points represent the mean corrected absorbance (±SD) of quadruplicate measurements. The reciprocal of the dilution factor used to dilute the plasma samples is reported on the x-axis. (B) Indirect ELISA against PSA-ACT. Plasma samples from 212 randomly selected samples were tested together with plasma from subject 1 to determine the frequency of autoantibodies. Data points represent the mean corrected absorbance of duplicate measurements expressed as a percentage of the mean absorbance for subject 1. Samples that displayed a reactivity beyond the 99% CI of the population are highlighted in red. S1: subject 1. (C) Indirect ELISA against PSA, PSA-ACT and ACT. Samples A to G are the samples highlighted in red in panel B. S1: subject 1. CT pool: control pool.

levels being as high or even higher than total PSA levels may hence serve as an indication of interference. Low or even undetectable total-PSA with high levels of free-PSA due to possible presence of autoantibodies has been previously reported [30, 31]. This erroneous phenomenon was not present in all tested PSA immunoassays, suggesting dependence on the selected PSA-antibodies used [30].

In our study, the autoantibody interfered with the measurement of PSA by nine investigated immunoassays, however to very different degrees with recoveries ranging from less than 10% to about 80%. Interestingly, our follow-up study identified the presence of PSA autoantibodies in >3% of patient sera from our hospital. Previously reported prevalences of anti-PSA antibodies amounted to 0% in young healthy males and females, 16% and 40% in infertile men and women, respectively, and 33% and 59% in men with benign prostate hypertrophy [13, 14, 32]. Importantly however, none of the autoantibodies identified in our prevalence study interfered with the determination of PSA by the Roche Assay, even when their titres were higher than that in the sample of Patient 1.

Several different prominent epitopes have been identified within the PSA molecule. These various epitopes may react differently with the antibodies used by the different PSA assays [33]. Van Duijnhoven et al. reported a case report on large discrepancies between PSA levels measured by different assays, possibly due to the presence of PSA autoantibodies. They discussed possible reasons and concluded that the PSA molecule of this specific patient may have an alternative structure, leading to a diminished recognition by the anti-PSA antibodies used in the affected assays, and may have a reduced binding of ACT [34].

Obviously only auto-antibodies with a specific epitope possibly shared with the antibodies of the PSA immunoassay interfere with the PSA immunoassay.

Likewise only a minority of anti-TG antibodies interferes with the measurement of TG so that several guidelines on the monitoring of thyroid cancer patients recommend the determination of TG recovery instead of or in addition to the determination of anti-TG antibodies [19].

Unrecognized interference of hA or anti-PSA autoantibodies with the PSA assay can lead to severe clinical

consequences. Given the wide clinical use of PSA assays in many countries and medical disciplines, a prevalence of interfering hA or anti-PSA antibodies amounting to 4 in 1158 puts a relevant number of men at risk of under- or over-diagnosis. As an important limitation, our study does not allow any conclusion whether prevalences of interfering hA's and anti-PSA autoantibodies vary among different patient groups and/or PSA assays. It may well be that the prevalence of these interferences with PSA measurement is affected by the specific immunoassay used or the presence or therapy of prostate cancer, or by other diseases. Nevertheless, our study adds important information about the prevalance of PSA disturbance in a large unselected male population. It raises the question of whether PSA measurements like TG measurements should be controlled by the determination of recovery.

Conclusions

We conclude that the prevalence of PSA measurements interfered by hA's or anti-PSA-autoantibodies is about 0.3%. Because of the high incidence of PSA testing, this rate puts a significant number of men at risk for a misinterpretation of their PSA test results.

In case of implausibly high or low PSA levels, the test should be repeated with a different PSA assay. Further work-up should also include the determination of PSA recovery. If the recovery is pathological, hA's and anti-PSA antibodies are prime candidates for underlying causes.

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